Nuclear Factor-κB-inducing Kinase (NIK) Contains an Amino-terminal Inhibitor of Apoptosis (IAP)-binding Motif (IBM) That Potentiates NIK Degradation by Cellular IAP1 (c-IAP1)*

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Background: NIK stability is necessary for activation of the noncanonical NF-κB pathway.
Results: The N terminus of NIK harbors an IBM that binds to c-IAP1 and promotes NIK degradation.
Conclusion: The NIK IBM provides substrate recognition for c-IAP1 activity to maximize the suppression of noncanonical NF-κB signaling.
Significance: This study reveals new mechanistic insight into the regulation of the c-IAP·TRAF2·TRAF3·NIK complex.

Activation of the noncanonical NF-κB pathway hinges on the stability of the NF-κB-inducing kinase (NIK), which is kept at low levels basally by a protein complex consisting of the E3 ubiquitin ligases cellular inhibitor of apoptosis 1 and 2 (c-IAP1/2) proteins and the tumor necrosis factor receptor-associated factors 2 and 3 (TRAF2/3). NIK is brought into close proximity to the c-IAPs through a TRAF2-TRAF3 bridge where TRAF2 recruits c-IAP1/2 and TRAF3 binds to NIK. However, it is not clear how the c-IAPs specifically recognize and ubiquitylate NIK in the complex. We have identified an IAP-binding motif (IBM) at the amino terminus of NIK. IBMs are utilized by a number of proapoptotic proteins to antagonize IAP function. Here, we utilize mutational studies to demonstrate that wild-type NIK is destabilized in the presence of c-IAP1, whereas the NIK IBM mutant is stable. NIK interacts with the second baculovirus IAP repeat (BIR2) domain of c-IAP1 via the IBM, and this interaction, in turn, provides substrate recognition for c-IAP1 mediated ubiquitylation and degradation of NIK. Furthermore, in the presence of the NIK IBM mutant, we observed an elevated processing of p100 to p52 followed by increased expression of NF-κB target genes. Together, these findings reveal the novel identification and function of the NIK IBM, which promotes c-IAP1-dependent ubiquitylation of NIK, resulting in optimal NIK turnover to ensure that noncanonical NF-κB signaling is off in the absence of an activating signal.

The transcription factor, nuclear factor-κB (NF-κB),[3] plays an important role in the maintenance and activation of leuko-cytes following an immune response (1). Thus, tight control of NF-κB is critical for the proper homeostatic function of many processes of innate immunity, especially the inflammatory response, and for activation of B and T cells during the adaptive immune response. Gene regulation by the NF-κB family of transcription factors is accomplished through differential dimerization of five discrete NF-κB subunits known as RelA, RelB, c-Rel, p50/p105, and p52/p100, which homo-and/or heterodimerize to form active transcription factors with different target gene specificities (2). Regulated processing of the larger precursor proteins p105 and p100 yield the transcriptionally functional p50 and p52 subunits, respectively. In quiescent cells, preformed NF-κB dimers are sequestered in the cytoplasm by a family of NF-κB inhibitory proteins (IκB). Nuclear translocation of the NF-κB dimers is governed by one of two regulatory kinase cascades, depending on the stimulus, referred to as the canonical and noncanonical pathways of NF-κB signaling.

Activation of the canonical pathway occurs downstream of the Toll-like receptors, the T and B cell receptors, and tumor necrosis factor receptors 1 and 2 (3, 4). After the cell receives a stimulus, IκB is targeted by an IκB kinase (IKK) complex consisting of IKKα, IKKβ, and NF-κB essential modulator (NEMO), leading to ubiquitylation and proteasomal degradation of IκB, allowing for nuclear translocation of RelA-p50 and cRel-p50 dimers. Alternatively, the noncanonical NF-κB pathway is activated downstream of a subset of TNF receptor family members, including LTβR, BAFFR, CD40, and CD30 (5–7). NF-κB-inducing kinase (NIK) is required for stimulation of the noncanonical NF-κB pathway and, under basal conditions, is continuously targeted for proteasomal degradation by an E3 ubiquitin ligase complex consisting of the cellular inhibitor of apoptosis 1 and 2 (c-IAP1 and c-IAP2) proteins and the TNF receptor-associated factors 2 and 3 (TRAF2 and TRAF3) (8, 9).

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[3] The abbreviations used are: NF-κB, nuclear factor-κB; IκB, inhibitor of κB; IKK, IκB kinase; NIK, NF-κB inducing kinase; c-IAP, cellular inhibitor of apo-ptosis; DHFR, dihydrofolate reductase; TRAF, tumor necrosis factor receptor-associated factor; BIR, baculovirus IAP repeat, XIAP, X-linked IAP; IBM, IAP-binding motif; Smac, second mitochondrial activator of caspases; Ub, ubiquitin.

Initiation of noncanonical NF-κB signaling results in disassembly of the complex and accumulation of NIK through c-IAP-mediated auto-degradation or degradation of TRAF2 and TRAF3, depending on the activating receptor (10). Stabilized NIK takes an active conformation, without the need for phosphorylation, and proceeds to phosphorylate and activate IKKα (11). In turn, IKKα phosphorylates the carboxyl-terminal IκB domain of the p100 precursor protein resulting in processing to allow p52-RelB dimers to translocate to the nucleus (12).

IAP proteins are members of a family defined by the presence of one or more baculovirus IAP repeat (BIR) domains (13, 14). Although the importance of IAPs in immune signaling is now appreciated, they were initially described, as their name implies, as modulators of apoptotic signaling (14). Most notably, X-linked IAP (XIAP) was found to selectively bind to and directly inhibit the proteolytic activities of caspases-3, -7 and -9 through its linker-BIR2 and BIR3 domains, respectively (14). In each instance, initial cleavage of the intersubunit linker, separating the large and small subunits of the caspase, resulted in the exposure of a neoepitope on the N terminus of the small subunit, known as an IAP binding motif (IBM), that inserted into a pocket in the BIR domain (14). Similar IBMs are present within many IAP-binding proteins, most of which share no homology outside of a conserved N-terminal tetrapeptide sequence that is exposed during protein maturation. Similar to XIAP, c-IAPs also contain BIR domains that can bind to caspases, but rather than directly inhibiting their catalytic activities, they instead efficiently ubiquitylate caspases and target them for destruction via the proteasome (15, 16). When overexpressed, c-IAPs may also block cell death by acting as sinks for IAP antagonists such as second mitochondrial activator of caspases (Smac; also known as DIABLO), which generally promote apoptosis by displacing active caspases from XIAP (17–20).

As alluded to above, the majority of the IAP proteins also display intrinsic E3 ubiquitin ligase activity via their RING domains (16, 21–23), and it is this activity that is thought to mediate their crucial roles in various signal transduction pathways. The E3 ubiquitin ligase activity of c-IAPs can also be modulated by their interactions with IBM-containing proteins (24). For example, during apoptosis, the release of mature Smac from mitochondria into the cytoplasm facilitates the interaction of the Smac IBM with IAPs. In addition to displacing caspases from IAPs, Smac (or Smac mimetic drugs) also promotes autoubiquitylation of c-IAP1/2, which stabilizes NIK and activates the noncanonical NF-κB signaling pathway (24, 25). In the current study, we have identified the presence of an IBM sequence at the N terminus of NIK and demonstrate that the NIK IBM is necessary for efficient c-IAP1-dependent ubiquitylation of NIK. Thus, the directed targeting of NIK ensures its maximal degradation, in turn suppressing downstream p100 processing and the transcriptional activation of NF-κB target genes. These findings are significant as they define a key regulatory element of the c-IAP·TRAF2·TRAF3·NIK complex.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Human embryonic kidney (HEK) 293T cells were grown in DMEM supplemented with 10% fetal bovine serum (Atlas Biologicals, Inc.), 2 mM Glutamax (Invitrogen), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were maintained at 37 °C and 5% CO_2.

Antibodies and Immunoblotting—Cell lysates were prepared by incubating cells on ice in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF, and 1 mM DTT) supplemented with complete mini protease inhibitor tablets (Roche Diagnostics). Protein samples were normalized by Bradford assay (Bio-Rad), resolved on denaturing polyacrylamide gels, and transferred to nitrocellulose membrane (Whatman). Membranes were blocked with 5% milk (w/v) in TBS containing 0.1% Tween 20 (Fisher) and incubated with the specified primary antibodies, washed, and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG or anti-rabbit IgG secondary antibodies (GE Healthcare). Peroxidase activity was detected by using enhanced chemiluminescence (GE Healthcare). Equal loading of protein samples was confirmed by blotting membranes with β-actin antibody. Antibodies used for immunoblotting: NIK (H-248), TRAF2 (C-20), TRAF3 (G-6), GST (Z-5) (Santa Cruz Biotechnologies); TRAF2 (BD Pharmingen); FLAG-HRP, HA-HRP, FLAG M2, and β-actin (Sigma); GFP (Novus).

Plasmids—To ensure exposure of the NIK IBM in our ectopic expression experiments, we generated a DHFR-HA-Ub-NIK-EGFP fusion construct as follows. First, using the pDhaUbXeK1βgal plasmid as template (kindly provided by A. Varshavsky), a cDNA for mouse dihydrofolate reductase (DHFR) fused to an HA tag and a ubiquitin (K48R) moiety was obtained by PCR and cloned into the NheI/BglII sites of pEFGP-N1 (26). Full-length NIK was then cloned into BglII/EcoRI, and the BglII site was removed by site-directed mutagenesis to ensure that glycine 76 of ubiquitin immediately preceded alanine 2 of NIK with no intervening sequences, thereby ensuring that deubiquitinases would cleave DHFR-HA-Ub from the NIK-EGFP during translation and expose alanine 2 of NIK. The NIK IBM mutant (A2G/V3G) was then generated by QuikChange site-directed mutagenesis (Agilent Technologies) following the manufacturer's instructions and using primer sequences: Ub-MNIK (A2G/V3G)-GFP, 5'-GAG GTG GTA TGG GAG GGA TGG AAA TGG-3' (forward) and 5'-CCA TTT CCA TCC CTC CCA TAC CAC CTC-3' (reverse); and Ub-NIK (A2G/V3G)-GFP, 5'-CTA AGA GGT GGT GGA GGG ATG GAA ATG-3' (forward) and 5'-CAT TTC CAT CCC TCC ACC ACC TCT TAG-3' (reverse). For expression of the individual BIR domains, BIR1, -2, and -3 of c-IAP1 were amplified by PCR using Phusion polymerase (New England Biolabs), and the resultant PCR products were subcloned into pEBG. The amplification and cloning of the BIR domains have been described (27). pEBB FLAG-cIAP1, pEBB FLAG-cIAP1 (H588A), pEBB c-IAP1-HA, pEBB cIAP1 (H588A)-HA, pEBB FLAG-Ub, Ub-Smac-HA, and Ub-Smac (A1G)-HA have been described (25). pEBB FLAG-NIK and pEBB NIK-FLAG were constructed by subcloning the coding sequence of NIK into the respective pEBB vector.

Plasmid Transfection and RNA Interference—HEK 293T cells were transfected with the indicated plasmid(s) and/or short interfering RNA using a standard calcium phosphate precipitation protocol (see figure legends for nucleic acid concentra-

tions). Suppression of TRAF3 with siRNA has been described previously (28). Proteasome inhibition was performed in the indicated experiments by treating the cells with 10 μM MG132 (Sigma) for 2–6 h. Dimethyl sulfoxide treatment was performed as a vehicle control.

Fluorescence Microscopy—HEK 293T cells were plated in glass-bottomed culture dishes (MatTek) at 50% confluence and were co-transfected with 0.3 μg of plasmids encoding either the wild-type or IBM mutant of NIK, each tagged with eGFP, together with 0.3 μg of FLAG-cIAP1. After 24 h of incubation, cells were stained with Hoechst (Sigma) for 20 min before analysis by fluorescent microscopy (Nikon ECLIPS Ti). To obtain percent GFP expression, pictures were taken for three fields of view in each culture dish, and the number of GFP-positive cells was divided by the total number of cells in each field.

Co-immunoprecipitation and Glutathione S-Transferase (GST) Pulldown—Cells were lysed by radioimmune precipitation assay buffer supplemented with complete mini protease inhibitor tablets (Roche Diagnostics) and incubated on ice for 30 min. The debris was removed by spinning cells down at 20,817 × g, 4 °C for 10 min. Normalized cell lysates were incubated with indicated antibodies and rotated for 2 h at 4 °C followed by the incubation with 30 μl of 50% slurry of protein G beads (Invitrogen) for 1 h. Beads were washed four times with 1 ml of radioimmune precipitation assay buffer, and protein complexes were eluted by 30 μl of 2× lithium dodecyl sulfate buffer (Invitrogen). The eluate was analyzed by SDS-PAGE followed by Western blotting. For GST pulldown, GST-tagged proteins were overexpressed in HEK 293T cells, and cells were lysed after 24 h of incubation. Radioimmune precipitation assay buffer cell lysates were prepared, as described for the co-IP experiments, and incubated with 40 μl of a 50% slurry of glutathione Sepharose beads (GE Healthcare) by rotating for 2 h at 4 °C. Beads were eluted and eluate analysis performed as described above for co-IP experiments.

Ubiquitin Conjugation Assay—FLAG-tagged ubiquitin was co-transfected with NIK and c-IAP1 plasmids into HEK 293T cells. The NIK antibody was used for immunoprecipitation, and final eluates were immunoblotted with an HRP-conjugated FLAG antibody to detect the level of ubiquitylated NIK.

Quantitative PCR—24 h post-transfection cell pellets were harvested and washed with PBS. Total RNA was extracted by using the Pure Link RNA mini kit (Ambion) according to the manufacturer's instructions. 100 ng of total RNA was subjected to a reverse transcription reaction using random hexamer primers and TaqMan MultiScribe Reverse Transcriptase (Applied Biosystems). The resulting cDNA was analyzed with the indicated TaqMan probe using the Bio-Rad CFX96 real-time PCR detection system. Each target assay was normalized to glyceraldehyde-3-phosphate dehydrogenase levels and performed in triplicate.

RESULTS

NIK Possesses an N-terminal IBM That Promotes NIK Degradation—To better understand the regulation of the c-IAP-TRAF-NIK complex, we searched for previously uncharacterized motifs and domains in NIK. Interestingly, we identified a putative IBM at the N terminus of NIK, just downstream of the initiating methionine, through sequence homology (Fig. 1*A*).

To test the function of the NIK IBM we mutated the position 2 alanine and position 3 valine, two amino acids that contribute significantly to the binding affinity of other IBMs to IAPs, to glycine (A2G/V3G) and asked whether NIK stability was affected in the presence of c-IAP1 (29). Wild-type (WT) NIK was degraded when ectopically expressed with c-IAP1 in HEK293T cells. However, NIK (A2G/V3G) was stabilized (Fig. 1*B*, compare *lanes 5* and *8*). As a control, a c-IAP1 RING mutant was used (H588A), which disrupts its E3 ubiquitin ligase activity and, as expected, c-IAP1 (H588A) did not show differential effects on the stability of WT or IBM mutant NIK. Similar results were obtained using c-IAP2, but it was relatively unstable compared with c-IAP1, resulting in a less dramatic affect on NIK stability (data not shown). We therefore focused our attention on c-IAP1. These results suggested that the NIK IBM was crucial for directing c-IAP1-mediated NIK degradation but were somewhat surprising since the initiator methionine in NIK would be predicted to block the c-IAP1-NIK IBM interaction. Thus, the initiator methionine of NIK was most likely removed, consistent with the propensity for methionine aminopeptidases to remove initiating methionines from proteins with less bulky amino acids in the second position (30). To test this possibility, we fused a FLAG tag to the N terminus of NIK (FLAG-NIK) to block the removal of the initiating methionine. FLAG-NIK was stable in the presence of c-IAP1, whereas a C-terminally FLAG-tagged NIK was unstable (Fig. 1*C*), further implying that the initiating methionine is removed to expose the NIK IBM.

To ensure processing of NIK and allow for full characterization of the NIK IBM without concern for whether or not the methionine was removed, we employed a system where ubiquitin is fused in-frame with the coding sequence for mature NIK (31). Deubiquitylating enzymes then co-translationally cleave off the ubiquitin molecule leaving the exposed NIK IBM. Using this system, we obtained similar results as those using full-length WT or IBM mutant NIK (Fig. 1. *B–D*). Additionally, an eGFP tag was fused to the carboxyl terminus of NIK to allow for easy monitoring of NIK stability. As shown in Fig. 1*E*, there was a significant increase in GFP detection in the NIK IBM mutant transfected cells as opposed to low GFP detection in the WT NIK IBM samples, when co-expressed with c-IAP1.

Data to this point suggested that the presence of WT NIK IBM directed the c-IAP1 E3 ligase activity toward NIK, resulting in degradation of NIK. To further examine whether the NIK IBM triggered ubiquitin-directed proteasomal activity, HEK293T cells were transfected with NIK-GFP or NIK (A2G/V3G)-GFP, in combination with vector, WT c-IAP1, or c-IAP1 (H588A) as a control for c-IAP1-mediated NIK degradation. Notably, cells treated with MG132 to block proteasome function confirmed that NIK was indeed degraded by the proteasome in an IBM-dependent fashion (Fig. 1*F*, compare *lanes 2* and *8*). Furthermore, using an ubiquitin immunoprecipitation experiment, we found that upon mutation of the NIK IBM a dramatic reduction of NIK ubiquitylation occurred in the presence of c-IAP1 (Fig. 1*G*, compare *lanes 2* and *3*, *6*, and *7*). Taken together, these results show that the NIK IBM has a key role in promoting NIK proteasomal degradation mediated by c-IAP1 E3 ubiquitin ligase activity.
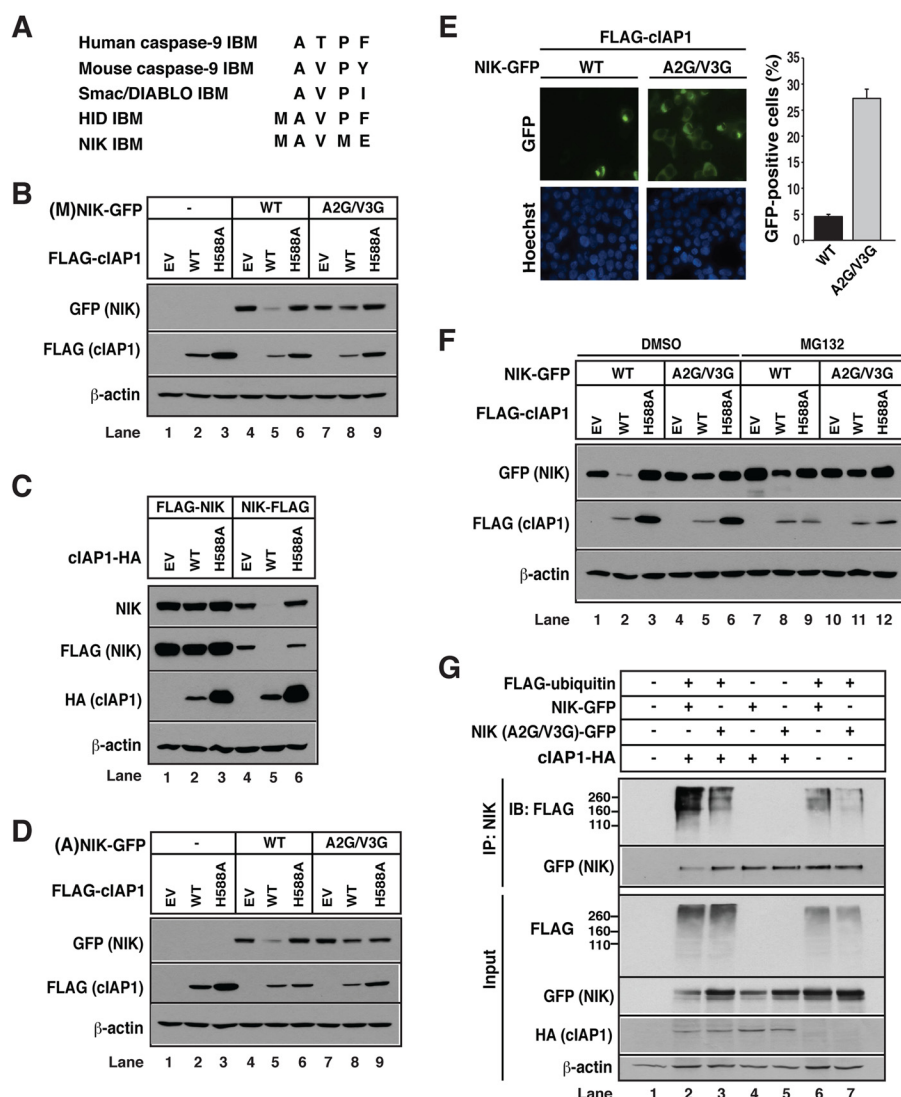
FIGURE 1. **NIK possesses an amino-terminal IBM that promotes NIK degradation.** *A*, alignment of the NIK IBM with other known IBMs of IAP-binding proteins. *HID,* head involution defective. *B*, plasmids encoding DHFR-Ub fused to WT NIK-GFP (0.5 μg) or NIK (A2G/V3G)-GFP (0.5 μg), including the initiating methionine (*M*), were transfected with plasmids encoding FLAG-cIAP1 (0.5 μg) in HEK293T cells. FLAG-tagged empty vector control (*EV*), WT, and E3 ligase mutant c-IAP1 (H588A) were used. After 24 h of incubation, the levels of NIK and c-IAP1 were determined by immunoblotting with anti-GFP and anti-FLAG-HRP, respectively. *C*, HEK293T cells were transfected with plasmids encoding either N-terminal or C-terminal FLAG-tagged NIK in the presence of vector control, WT c-IAP1, or the c-IAP1 E3 ligase mutant H588A. NIK protein levels were determined by both anti-NIK and anti-FLAG-HRP. HA-HRP antibody was used to detect the levels of c-IAP1. *D*, plasmids encoding DHFR-Ub fused to NIK-GFP or NIK (A2G/V3G)-GFP, without the initiating methionine, were transfected with plasmids encoding FLAG-cIAP1 or FLAG-cIAP1 (H588A) in HEK293T cells, and Western blots were performed as described in *B*. *E*, WT NIK-GFP or NIK (A2G/V3G)-GFP were co-expressed with WT c-IAP1 in HEK293T cells. The stability of NIK-GFP was analyzed by fluorescence microscopy, and the nuclei of cells were visualized by Hoechst staining. The graph indicates percent of GFP positive cells. *F*, plasmids encoding the indicated proteins were transfected into HEK293T cells, and NIK protein levels were detected by Western blot in the presence of either c-IAP1 or c-IAP1 (H588A). To inhibit proteasomal degradation, cells were treated with MG132 (10 μM) for 6 h. The levels of NIK and c-IAP1 were analyzed by anti-GFP and anti-FLAG-HRP, respectively. *G*, the level of NIK ubiquitylation was examined by an ubiquitin-conjugation assay after co-transfection of plasmids encoding FLAG-ubiquitin together with NIK-GFP or NIK (A2G/V3G)-GFP and cIAP1-HA in HEK293T cells. An NIK antibody was used to pulldown NIK followed by an immunoblot using FLAG-HRP antibody to visualize the ubiquitylated NIK. Anti-GFP and anti-HA-HRP were used to probe for NIK and c-IAP1, respectively. To inhibit proteasomal degradation, cells were treated with MG132 (10 μM) for 2.5 h before harvesting the cells.

The NIK IBM Is Important for Reinforcing the Interactions within the c-IAP1-TRAF2-TRAF3-NIK Complex and Is Necessary for Proper E3 Ubiquitin Ligase Targeting of NIK by c-IAP1—Based on previous studies, it is known that suppressing TRAF3 stabilizes NIK by breaking the TRAF3 bridge with TRAF2, the adaptor protein responsible for c-IAP recruitment (28, 32–35). Thus, we overexpressed either the mature WT NIK-GFP or NIK (A2G/V3G)-GFP, along with control siRNA or siRNA targeted to endogenous TRAF3. Cells were also transfected with vector control, WT c-IAP1, or the E3 ligase (H588A) mutant as

a control for NIK degradation. Prior to TRAF3 suppression, NIK-GFP and TRAF3 were degraded in the presence of WT c-IAP1, whereas, as expected, NIK was stabilized after depletion of TRAF3 (Fig. 2*A*, compare *lanes 2* and *5*). The NIK IBM mutant, however, was stable irrespective of TRAF3 expression (Fig. 2*A*, compare *lanes 2* and *5* with *8* and *11*). Collectively, these results strongly suggested that the NIK IBM contributed to proper formation of the c-IAP1·TRAF2·TRAF3·NIK protein complex.

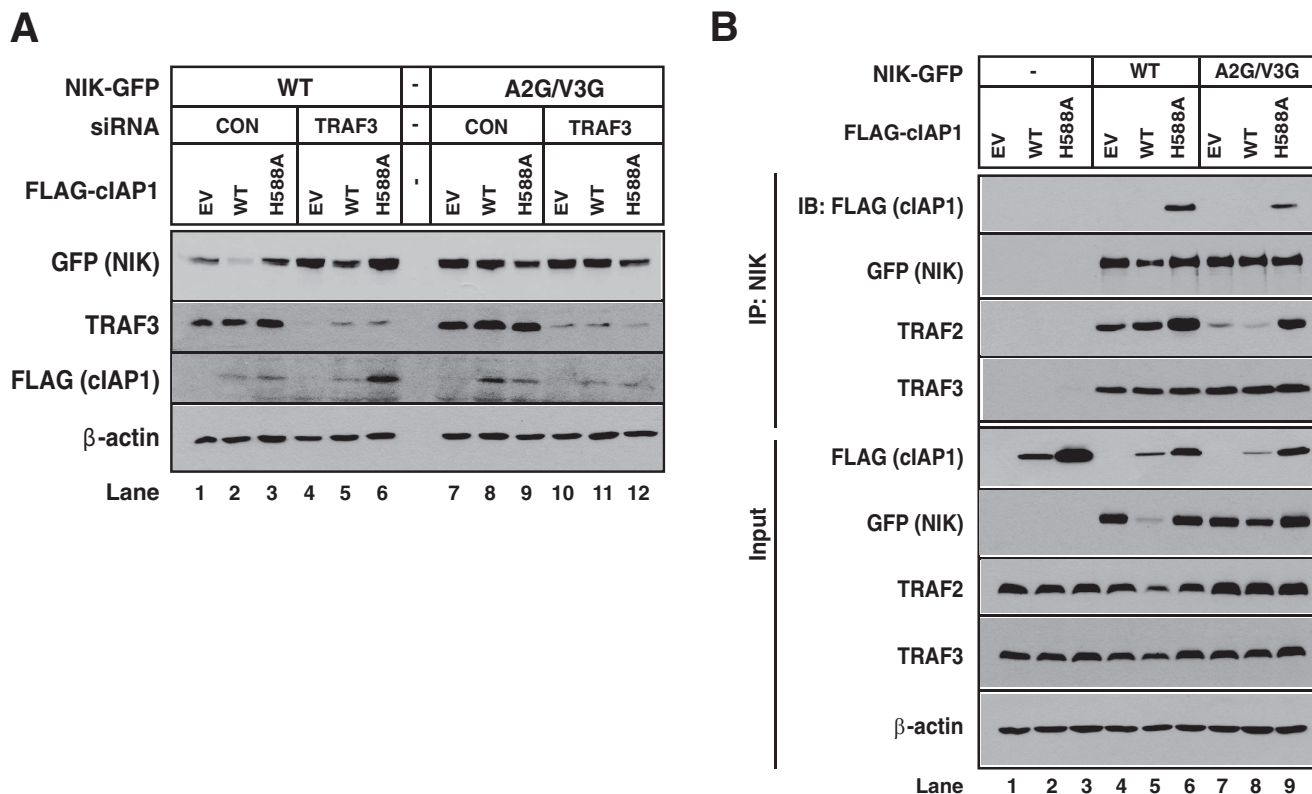To further study the role of the NIK IBM in complex formation, NIK-GFP or NIK (A2G/V3G)-GFP was expressed in HEK293T

FIGURE 2. The NIK IBM is important for reinforcing the interactions within the c-IAP·TRAF2·TRAF3·NIK complex and is necessary for proper E3 ubiquitin ligase targeting of NIK by c-IAP1. *A*, HEK293T cells were transfected with TRAF3 siRNA to suppress endogenous TRAF3 expression along with plasmids encoding NIK-GFP or NIK (A2G/V3G)-GFP and FLAG-cIAP1 or FLAG-cIAP1 (H588A). Cell lysates were prepared after 48 h of incubation and analyzed by Western blot using anti-GFP, anti-TRAF3, and anti-FLAG-HRP. *B*, FLAG-cIAP1 constructs with either NIK-GFP or NIK (A2G/V3G)-GFP were transfected in HEK293T cells, and co-IPs were conducted using an anti-NIK antibody. Eluates were immunoblotted by anti-FLAG-HRP for detection of the c-IAP1-NIK interaction. In addition, the levels of endogenous TRAF2 and TRAF3 in the c-IAP·TRAF·NIK complex were also analyzed by Western blot. The expression levels of NIK, cIAP1, TRAF2, and TRAF3 in input lysates were confirmed by immunoblotting with indicated antibodies. *CON*, control; *EV*, empty vector.

cells, along with either empty vector, WT c-IAP1, or c-IAP1 (H588A), followed by immunoprecipitation of NIK to isolate the c-IAP1·TRAF2·TRAF3·NIK complex (Fig. 2*B*). Interestingly, the endogenous TRAF proteins, along with WT NIK-GFP, were partially degraded following co-expression with WT c-IAP1, presumably by NIK IBM-directed c-IAP1 activity (Fig. 2*B*, *lane 5*, *inputs*). However, the small amount of WT NIK-GFP remaining was sufficient to co-immunoprecipitate the residual endogenous TRAF2 and TRAF3 (Fig. 2*B*, *lane 5*, *IPs*). In contrast, NIK (A2G/V3G) was unable to bind TRAF2 but maintained a strong interaction with TRAF3 (Fig. 2*B*, compare *lanes 5* and *8*). Because WT c-IAP1 was unstable and difficult to detect following co-immunoprecipitation (co-IP) with WT NIK (Fig. 2*B*, *lane 5*), we also utilized the E3 ligase mutant c-IAP1 (H588A) to stabilize its expression in cells. In doing so, compared with WT NIK, the NIK IBM mutant exhibited a significant decrease in binding to c-IAP1 (H588A) and its adapter TRAF2, but not to TRAF3 (Fig. 2*B*, compare *lanes 6* and *9*). Altogether, these findings suggest that the NIK IBM further stabilizes the interactions within the c-IAP1·TRAF2·TRAF3·NIK complex apart from the TRAF bridge. Thus, the NIK IBM *per se* appears to mediate and perhaps even stimulate c-IAP1-dependent turnover of NIK within the complex; in its absence, c-IAP1 instead targets TRAF2 and itself for ubiquitylation and turnover. This finding is reminiscent of previous work in *Drosophila*, wherein DIAP1 targets BIR2-bound substrates (possessing

IBMs) for ubiquitylation and turnover, but in the absence of bound substrates, instead targets itself for destruction (36).

The NIK IBM Interacts with BIR2 of c-IAP1 in a Fashion Similar to That of the Smac IBM—It is well established that IBMs bind specifically to a unique groove in the BIR domains of IAP proteins (16, 18, 19, 29, 37). To further support our hypothesis that the amino terminus of NIK is an IBM, and to identify the BIR domain(s) to which NIK binds, we performed pulldown experiments with NIK and the BIR regions of c-IAP1 (Fig. 3*A*). Interestingly, although mature Smac bound to BIR2 and BIR3 of c-IAP1, as expected, NIK bound specifically to BIR2 and mutation of the NIK IBM disrupted this interaction (Fig. 3, *B* and *C*). Remarkably, NIK did not interact with XIAP, regardless of the IBM status, whereas Smac interacted with XIAP in an IBM-dependent manner (Fig. 3*D*). These results reveal that the NIK IBM recognizes a specific BIR domain of c-IAP1 and suggests that the modest interaction observed between NIK (A2G/V3G) and c-IAP1 (H588A) (Fig. 2*B*) is a consequence of indirect binding mediated through the TRAF bridge.

To investigate the binding nature of the NIK IBM to c-IAP1, NIK associations with c-IAP1 were monitored by co-IP in the presence of increasing concentrations of mature Smac. Titration of mature Smac, even at the lowest transfected plasmid concentration, competitively hindered NIK from binding to c-IAP1, which was dependent on the Smac IBM (Fig. 4, *A* and *B*). To confirm that NIK was in fact interacting with the BIR2
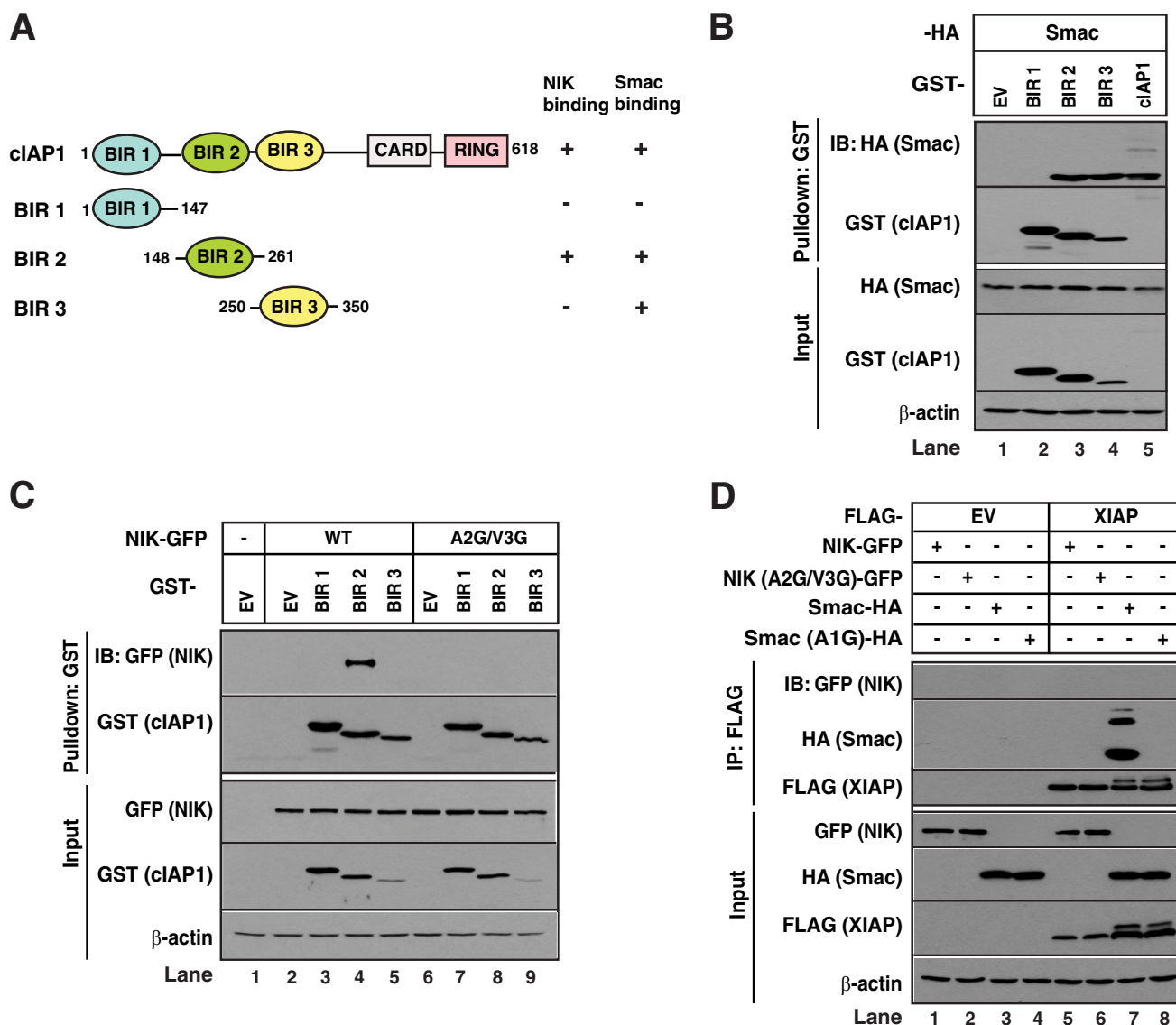
FIGURE 3. **NIK IBM interacts with the BIR2 domain of c-IAP1.** *A*, diagram of c-IAP1 constructs used in this study. The WT full-length c-IAP1 and each of the three BIR domains of c-IAP1 were fused to GST. *CARD*, caspase recruitment domain; *RING*, really interesting new gene. Interactions of BIR domains with NIK and Smac are described as the presence (+) or absence (−) of interaction. *B*, Smac-HA was ectopically expressed along with the individual GST-tagged BIR domains or full-length c-IAP1 in HEK293T cells. Glutathione-Sepharose (*GSH*) beads were used to pull down the BIRs of c-IAP1, and the eluates were probed with anti-HA-HRP to determine the presence of Smac. *C*, NIK-GFP or NIK (A2G/V3G)-GFP was co-expressed with each individual GST-BIR domain in HEK293T cells, and GSH beads were used to pulldown GST. The levels of NIK and BIR domains were confirmed by immunoblotting (*IB*) with anti-GFP and anti-GST, respectively. *D*, plasmids encoding NIK-GFP, NIK (A2G/V3G)-GFP, Smac-HA, Smac (A1G)-HA, and FLAG-XIAP were transfected in HEK293T cells as indicated. Cell lysates were incubated with anti-FLAG to analyze the interaction of XIAP with either NIK or Smac by co-IP.

binding pocket of c-IAP1, we mutated two key residues within the groove, aspartate 234 and glutamate 239, to alanine in the context of WT c-IAP1 (D234A/E239A) or the H588A mutant (D234A/E239A/H588A) (38). We then assessed NIK binding via co-IP, and as anticipated, found that NIK could no longer bind to c-IAP1 (D234A/E239A/H588A) and was instead stabilized (Fig. 4C, compare *lanes 8* and *10*, IP and *lanes 7* and *9*, *Input*). Combined, these data argue that NIK selectively targets distinct BIR domains within specific IAP proteins, binding in an IBM-dependent manner.

The NIK IBM Ensures Maximal NIK Degradation to Keep Noncanonical NF-κB Signaling Off—Stabilization of NIK leads to the phosphorylation and processing of p100. Thus, we examined whether NIK (A2G/V3G)-GFP could also activate the nonca-

nonical pathway. Co-expression of NIK-GFP alone with FLAG-p100-HA in HEK293T cells yielded the active p52 NF-κB subunit that was not present in the absence of NIK (Fig. 5A, compare *lanes 2* and *5*). The addition of c-IAP1 resulted in NIK degradation as shown above and diminished accumulation of p52 (Fig. 5A, compare *lanes 5* and *6*). However, mutation of the NIK IBM reestablished p100 processing in the presence of c-IAP1 (Fig. 5A, compare *lanes 6* and *9*), confirming that the NIK IBM is vital for maintaining noncanonical signaling in an off state. These results suggested that disruption of the NIK IBM might drive continuous noncanonical NF-κB activity, independent of membrane receptor-stimulation. To address this possibility, NIK or NIK (A2G/V3G) was expressed in the absence or presence of c-IAP1 after which the expression of
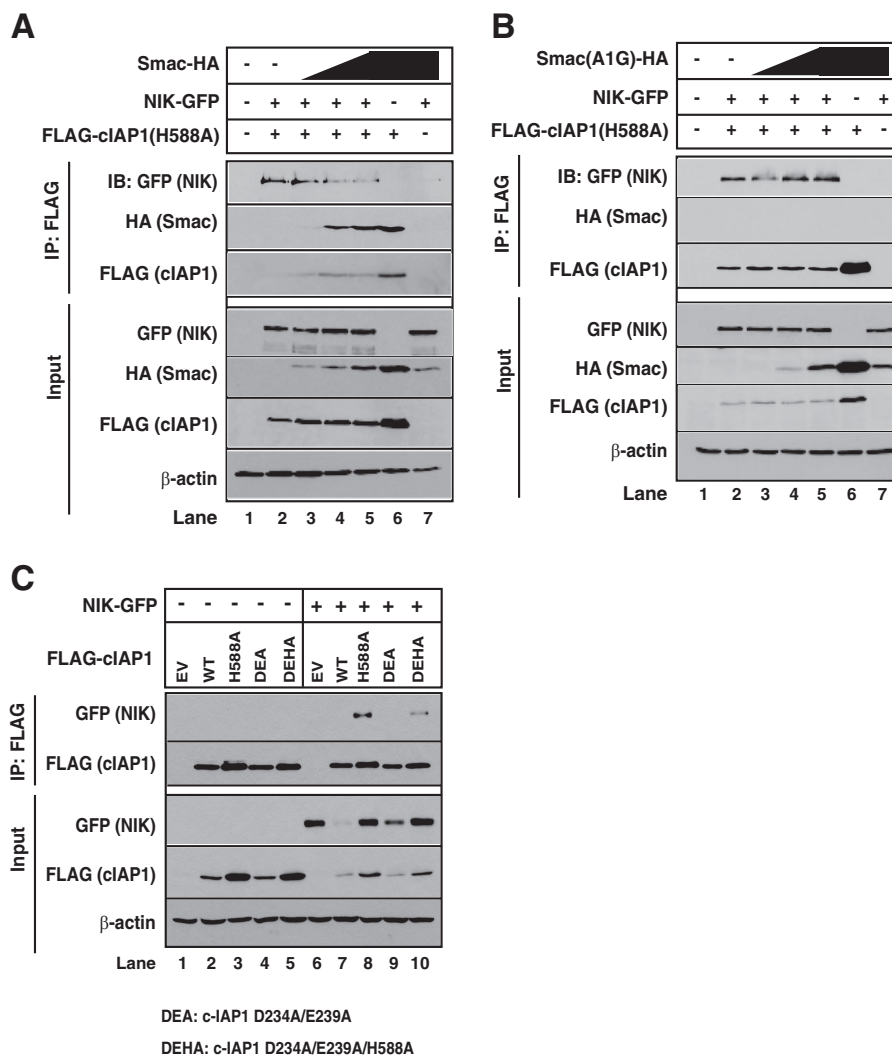
FIGURE 4. **NIK and Smac compete for the binding groove in c-IAP1 BIR2.** *A* and *B*, interaction between NIK-GFP (2 μg) and FLAG-c-IAP1 (H588A) (2 μg) was examined by co-IP in the presence of increasing amounts of ubiquitin-fused Smac-HA (0.5 μg, 1 μg, 2 μg) to interrogate the competitive interactions between NIK and Smac for c-IAP1. Anti-FLAG was used to IP c-IAP1, and anti-GFP and anti-HA-HRP were used to analyze the eluates for the presence of NIK and Smac, respectively. Mature Smac and the Smac IBM mutant (A1G) were similarly titrated. *C*, plasmids encoding FLAG-tagged WT c-IAP1, H588A, D234A/E239A, or D234A/E239A/H588A were transfected along with a plasmid encoding DHFR-Ub fused to NIK-GFP in HEK293T cells. A co-IP was performed by using anti-FLAG, and both eluates and input lysates were analyzed by anti-GFP and anti-FLAG-HRP to confirm the levels of NIK and c-IAP1.

NF-κB target genes were monitored by quantitative PCR. Expression of NIK-GFP with c-IAP1 resulted in a 2-fold reduction in expression of the indicated NF-κB target genes, compared with expression of NIK with a vector control (Fig. 5*B*). Interestingly, mutation of the NIK IBM rescued the expression of these genes in the presence of c-IAP1 (Fig. 5*B*). Collectively, our data suggest that the NIK IBM facilitates c-IAP1-mediated degradation of NIK, which suppresses p100 processing and transcriptional up-regulation of noncanonical NF-κB target genes.

DISCUSSION

In this study, we have defined the function of an IBM at the amino terminus of NIK and shown that the NIK IBM is essential for reinforcing intermolecular interactions within the c-IAP·TRAF2·TRAF3·NIK complex to allow for proper degradation of the complex under basal conditions. These findings are significant as they shed light on the molecular switch that

regulates the stability of not only NIK, but also the TRAFs within the complex. Furthermore, our results reveal how loss of the NIK IBM, as occurs in certain translocation events, contributes to NIK stability. For instance, in a multiple myeloma cell line that harbors a NIK translocation that deletes the IBM and TRAF3 binding sites, NIK is still recruited into the c-IAP·TRAF2 complex through its interactions with TRAF2 but is not degraded (39). In turn, stabilized NIK activates noncanonical NF-κB signaling leading to cell survival and cancer growth, especially in B cell malignancies given the intimate integration of noncanonical signaling with B cell survival (40). This suggests that the NIK IBM is necessary to facilitate c-IAP1-mediated ubiquitylation of NIK by properly orienting NIK as the substrate.

Interestingly, it appears that the initiator methionine of NIK is removed given the fact that the WT NIK was degraded in the presence of c-IAP1, whereas an N-terminal FLAG-tagged NIK was stable. This is strikingly similar to how the IBM of the *Drosophila* IAP antagonists Head involution defective (Hid),
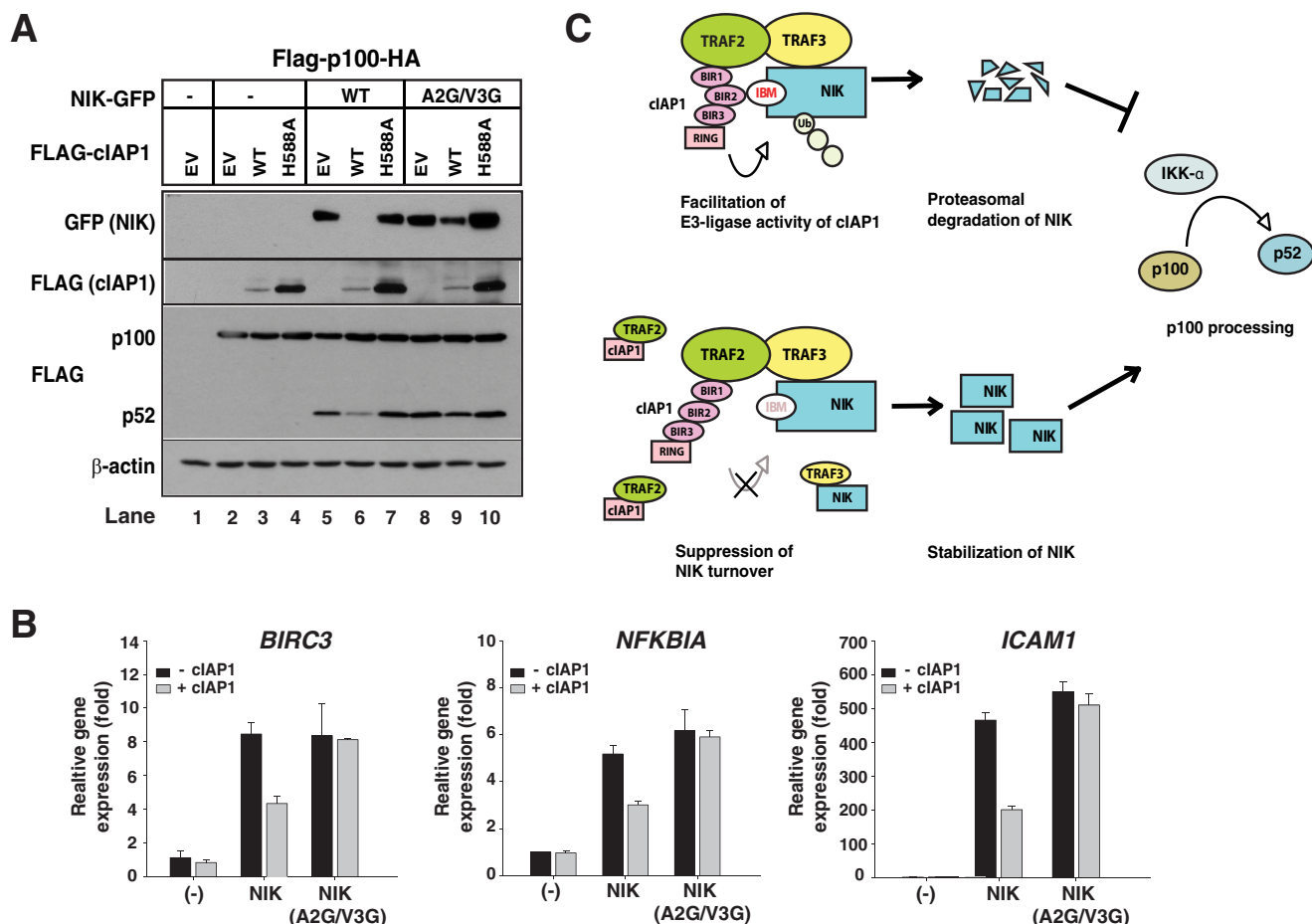
FIGURE 5. **The NIK IBM ensures maximal NIK degradation to keep noncanonical NF-κB signaling off.** *A*, plasmids encoding the indicated proteins were transfected into HEK293T cells followed by immunoblot analysis with the indicated antibodies to determine the affects on p100 processing when the NIK IBM was mutated. *B*, HEK293T cells were transfected with plasmids encoding DHFR-Ub fused to NIK-GFP or NIK (A2G/V3G)-GFP in the presence or absence of FLAG-cIAP1. After 24 h of incubation, cells were collected for mRNA extraction followed by cDNA generation. The mRNA expression levels of noncanonical NF-κB target genes (*BIRC3*, *NFKBIA*, *ICAM1*) were analyzed by quantitative real-time PCR using TaqMan probes. *C*, proposed model of the NIK IBM's role in regulating NIK stability and noncanonical NF-κB signaling. The NIK IBM promotes proteasomal degradation of NIK through c-IAP1-mediated ubiquitylation of NIK to suppress activation of the noncanonical NF-κB pathway. Loss or mutation of the NIK IBM leads to partial dissociation of the c-IAP-TRAF-NIK complex, NIK stabilization, and aberrant activation of noncanonical NF-κB signaling.

Reaper (Rpr), and Grim are exposed, suggesting that NIK is processed in a similar fashion (36, 37, 41, 42). Once exposed, the NIK IBM binds to the second BIR of c-IAP1 to allow for maximal NIK ubiquitylation and degradation in the absence of an activating signal (Fig. 5*C*). Intriguingly, we found that the NIK IBM also drives degradation of TRAF2 and TRAF3 within the complex. Because TRAF2 and TRAF3 are involved in other signaling platforms, the NIK IBM may have a role in the regulation of a number of signaling pathways. Subsequently, loss or mutation of the NIK IBM results in insufficient complex formation, NIK stabilization, activation of the noncanonical NF-κB pathway, and possibly modulation of other signaling pathways (Fig. 5*C*).

Notwithstanding the fact that we have not directly tested binding affinity in this study, the specific binding of the NIK IBM to BIR2 of c-IAP1, and the ability to compete away NIK binding to c-IAP1 with Smac, could imply that the NIK IBM may have low binding affinity. This prediction might help explain how NIK dissociates from the complex following an activating signal. Furthermore, an IBM with weak binding affinity would protect cells from spontaneous apoptosis, which

might occur after NIK stabilization if the NIK IBM could bind tightly to numerous BIR domains in various IAP proteins. However, the NIK IBM needs to be more closely interrogated to support these predictions.

Although our data suggest that the NIK IBM properly orients the component proteins in the c-IAP·TRAF complex to confer specificity for the E3 ubiquitin ligase activity of c-IAPs, NIK IBM binding to c-IAP1 may also play a role in activating the ligase activity of c-IAP1, although neither feature need be mutually exclusive. Indeed, c-IAP1 E3 ligase activity is tightly auto-regulated by a specific conformation in the inactive enzyme that places the CARD domain in close proximity to the RING (43). Thus, c-IAP1 must undergo conformational changes to become activated (43). Consequently, the NIK IBM may bind to BIR2 of c-IAP1 and induce a conformational change that activates c-IAP1, thereby leading to degradation of the c-IAP·TRAF2·TRAF3·NIK complex. Despite the fact that more work is necessary before this hypothesis can be fully addressed, there is precedence for endogenous IBM-containing, or small molecule, IAP antagonists inducing conformational changes in c-IAP1 that stimulate its E3 activity (44).

However, these scenarios lead to autoubiquitylation and proteasomal degradation of c-IAP1.

Although great strides have been made in the elucidation of noncanonical NF-κB regulatory paradigms, there is a need for clearer mechanistic studies into the regulation of NIK stability. In fact, comprehending how NIK protein levels are managed within the c-IAP·TRAF2·TRAF3·NIK complex is a major emphasis of research in the field (8). The identification and characterization of an IBM in the amino terminus of NIK, as shown here, provides an important step forward in our understanding of the regulation of NIK stability and of the noncanonical NF-κB signaling pathway as a whole.

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